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(71) Applicant *(for all designated States except US)*: MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: GUARENTE, Leonard, P. [US/US]; 72 Goodnough Road, Chestnut Hill, MA 02167 (US). AUSTRIACO, Nicanor, Jr. [US/US]; 54 Boston Avenue, Somerville, MA 02144 (US). CLAUS, James [US/US]; Apartment 112, Bexley Hall, Cambridge, MA 02139 (US). COLE, Francesca [US/US]; Apartment 205W, 108 Gainsborough Street, Boston, MA 02115 (US). KENNEDY, Brian [US/US]; 16 Laurel Street, Arlington, MA 02174 (US).

(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

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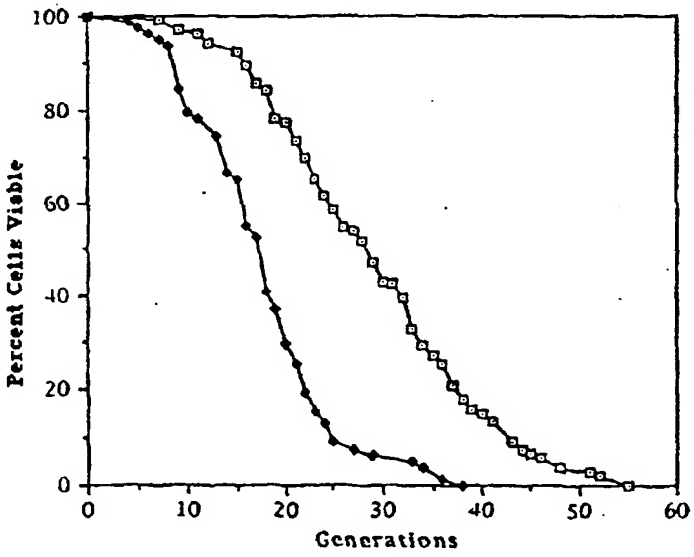

(57) Abstract

Methods of isolating mutant yeast cells with increased life span, as well as mutant yeast cells isolated by the methods, are disclosed. Also described are methods of identifying agents which increase life span of yeast cells, and methods of isolating genes which contribute to senescence in organisms.

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GENES DETERMINING CELLULAR SENESCENCE IN YEAST

Background of the Invention

Aging is a process in which all individuals of a species undergo a progressive decline in vitality leading to death. In metazoans, aging at the level of the whole organism is clearly evident. Whether the aging of an organism is genetically programmed, or represents the effects of entropy over time is not clear. Consistent with the possibility of a genetic program are mutations which alter the aging process. In humans the genetic diseases progeria and Werner's syndrome cause premature aging in affected individuals. In the earthworm *C. elegans*, a gene, age-1, has been described which directly or indirectly affects the life span of the animal (Friedman, D.B. and Johnson, T.E., Genetics 18:75-86 (1988)). A further issue open to speculation is how the aging of the entire organism relates to the aging of individual cells and cell types within the organism.

That individual cells within mammals do senesce was demonstrated in the findings of Hayflick, who showed that primary human diploid fibroblasts (HDFs) would grow in culture for about 50 population doublings, and then all the cells in the population would stop dividing (Hayflick, L. and Moorhead, P.S., Exp. Cell Res. 25:585-621 (1961); Hayflick, L., Exp. Cell Res. 37:614-636 (1965)). Cells arrest in the G1 phase of the cell cycle and contain a 2N chromosomal complement (Cristofalo, V.J., et al., Exp. Gerontol. 24:367 (1989)). This in phase, or clonal, senescence of the HDFs is accompanied by a characteristic morphological change; cells enlarge as they senesce (Angello, J.C., et al., J. Cell. Physiol. 132:125-130 (1987) and Cristofalo, V.J. and Kritchevsky, D., Med. Exp. 19:313-320 (1969)). In fact, this direct correlation between cell size and senescence can be demonstrated by

incubating young HDFs in low serum-medium, in which they enlarge, but do not leave the G1 phase of the cell cycle (Angello, J.C., et al., J. Cell. Physiol. 140:288-294 (1989)). When these cells are returned to medium containing adequate serum for cell division, their program of senescence has been advanced compared to smaller cells which have divided the same number of times.

Cell fusion studies between old and young HDFs indicate that senescence is dominant. In short term hybrids, initiation of DNA synthesis in the young nucleus is inhibited after the young cell has been fused to a senescent HDF (Norwood, T.H., et al., Proc. Natl. Acad. Sci. USA 71:2231 (1974)). In fact, injection of polyA+ RNA from the senescent HDF into the young cell inhibits DNA synthesis (Lumpkin, C.K., Jr., et al., Science 232:393 (1986)), suggesting that the senescent HDF activated a gene or genes that encoded dominant inhibitory proteins. In complementation studies that involve fusing various "immortal" cell lines, four genes were identified which were involved in immortalization (Pereira-Smith, O.M. and Smith, J.R., Proc. Natl. Acad. Sci. USA 785:6042 (1988)). The dominance of senescence appears to conflict with the view that shortening of telomeres, a phenomenon observed during passage of fibroblasts (Harley, C.B., et al., Nature 345:458 (1990)), causes senescence.

In several lower eukaryotes, senescence has been demonstrated and linked to changes in mitochondria. In *Podospora*, cell senescence is strongly associated with the excision and amplification of segments of mitochondrial DNA (Cummings, D.J., et al., J. Mol. Biol. 185:659-680 (1985) and Koll, F. et al., Plasmid 14:106-117 (1985)). In *Neurospora* (Bertrand J., et al., Cell 47:829-837 (1986)) and *Aspergillus* (Lazarus, C.M., et al., Eur. J. Biochem 106:663-641 (1989)), senescent cells also contain rearrangements in their mitochondrial DNA. In all of the

above examples, the senescent phenotype is dominant and is inherited cytoplasmically.

In the budding yeast, *Saccharomyces cerevisiae*, cells divide asymmetrically, giving rise to a large mother cell and a small daughter cell. By micromanipulating the daughter away from the mother at each cell division, it was shown that the mother divided a fixed number of times, and then stopped (Mortimer, R.K. and Johnston, J.R., Nature 183:1751-1752 (1959)). Life span was thus defined by the number of divisions mother cells had undergone, and not by chronological time. Further, a number of cell divisions in the life span of the mother, while fixed (varying over a Gompertz distribution (Pohley, J.-J. Mech. Ageing Dev. 38:231-243 (1987)), could differ from strain to strain (ranging from about 15 to 30) (Egilmez, N.K. and Jazwinski, S.M., J. Bacteriol. 171:37-42 (1989)). Thus, senescence in budding yeast as in HDFs is not a stochastic process, but has some underlying genetic basis.

Senescence in yeast is like senescence in HDFs in other ways as well. Like HDFs, yeast mother cells have been shown to enlarge with age (Mortimer, R.K. and Johnston, J.R., Nature 183:1751-1752 (1959) and Egilmez, N.K., et al., J. Gerontol. Biol. Sci. 45:B9-17 (1990)). In addition to their large size, aging mother cells also divide more slowly than young cells (Egilmez, N.K. and Jazwinski, S.M., J. Bacteriol. 171:37-42 (1989)). A further analogy to HDFs is that the senescent phenotype is also dominant in yeast. Mating a young yeast cell to an old one generates a diploid with a limited potential for cell division (Muller, I., J. Microbiol. Serol. 51:1-10 (1985)). In addition, daughters of old mothers display elongated cycling times for the first few divisions after separation from the old mother (Egilmez, N.K. and Jazwinski, S.M., J. Bacteriol. 171:37-42 (1989)). Evidently, the senescence substance is inherited by the

daughter cell and slowly degraded or diluted in subsequent cell cycles.

The senescence of yeast mother cells thus has similarities to what occurs in primary HDFs; however, there is one important difference. In yeast at each cell division the daughter cell has regained the capacity for a full life span, whether derived from a younger or older mother cell (Muller, I., Arch. Mikrobiol. 77:20-25 (1971)). This "resetting" in daughters may be intertwined with the mechanism that generates asymmetry at cell division. In any case, "resetting" argues against one category of hypothesis for aging; namely that aging results from the accumulation of errors in protein synthesis, the error catastrophe theory (Orgel, L.E. Nature 243:441 (1973)). Because daughter cells derived from old mothers have functional mitochondria (Muller, I. and Wolf, F., Mol. Gen. Genet. 160:231-234 (1978)), this resetting also shows that senescence is not due to rearrangements in the mitochondrial genome.

By varying the growth rate of cells, it was demonstrated that the key parameter in determining the life span in yeast is number of divisions, and not chronological time (Muller, I., et al., Mech. Ageing Dev. 12:47-52 (1980)). This finding led to the idea that senescence could be due to an accumulation of bud scars in mother cells. Bud scars are deposits of chitin that stay with the mother cell after each cell division (Cabib, E., et al., Curr. Top. Cell. Regul. 8:1-32 (1974), and Pringle, J.R., et al., Meth. Cell Biol. 31:357-435 (1989)). Several lines of evidence have argued against the idea that bud scars cause aging. First, varying the surface to volume ratio of isogenic yeast strains by varying their ploidy did not affect life span (Muller, I., Arch. Mikrobiol. 77:20-25 (1971)). Second, increasing the surface area by mating an old cell to a young one did not endow the diploid with an

increased potential for division (Muller, I., J. Microbiol. Serol. 51:1-10 (1985)). Third, induction of chitin synthesis and deposition in the cell wall did not decrease the life span of cells (Egilmez, N.K. and Jazwinski, S.M., J. Bacteriol. 171:37-42 (1989)). Thus, senescence in yeast has gross features similar to the aging process in mammalian cells. It is therefore reasonable to speculate that the molecular mechanisms of aging might be similar in yeast and mammalian cells, particularly in light of striking parallels in basic cellular mechanisms in yeast and mammalian cells. In the field of transcription, for example, there has emerged strong mechanistic similarities in the function of transcription factors: the yeast and mammalian TATA box binding factor TFIID, are interchangeable in the basal *in vitro* transcription reaction (Buratowski, S., et al., Nature 334:37-42 (1988)). Further, yeast and certain mammalian transcriptional activators will function normally in the heterologous host cells (see Guarente, L., et al., Cell 52:303-305 (1988) for review). Therefore, further study of aging in yeast cells may yield information concerning genes which are involved in senescence, and ultimately may shed light on the aging process in mammalian cells.

Summary of the Invention

The present invention pertains to the discovery that a particular gene contributes to senescence in eukaryotic cells, such as in budding yeast, and that a mutation in this gene contributes to a longer life span. As described herein, it was discovered that the SIR4 gene (silent information regulator) contributes to senescence: when mutant yeast cells are generated by a specific mutation in the SIR4 gene, the resultant mutant cells have a life span that is significantly longer than the life span of the non-

mutant strain. The mutation is an amber mutation that removes 121 residues from the 1358 residue SIR4 protein.

As a result of this discovery, methods of isolating mutant yeast cells with increased life span, and the mutant yeast cells isolated by these methods, are now available. Also available are methods to identify agents which enhance the life span of yeast cells; methods to isolate genes involved in senescence, as well as the genes isolated thereby, and the proteins encoded by the genes.

As described in detail below, the current invention comprises several methods of isolating yeast cells with increased life spans (a life span longer than the known life span of the non-mutagenized yeast strain). In each method, a sample of yeast cells from a budding yeast strain, for which the life span is known or has been calculated, is exposed to a mutagen, and then the mutagen-exposed yeast cells are cultured. In one embodiment of the current invention, mutant yeast cells are identified first by the related phenotype of starvation resistance. The yeast cells are plated on minimal medium, replica-plated on starvation medium, and grown. The plate with starvation medium is replica-plated to enriched medium; those colonies which grow are starvation resistant. The starvation-resistant colonies are then examined to isolate cells with longer life spans.

In a second embodiment, the cell surface of yeast cells are labelled with a fluorescent marker. New cells remain unlabelled. After a period of growth greater than the known life span of the yeast strain, the cells are subjected to fluorescence-activated cell sorting to isolate the fluorescent-labelled cells, which are then plated. Only those cells with longer life spans grow. In another embodiment, a temperature-sensitive budding yeast strain, in which the daughter cells die at the non-permissive temperature, is used. When cells from the temperature-

sensitive strain are grown at the non-permissive temperature, they form microcolonies in which the number of cells in the microcolony is equivalent to the number of generations in the life span of the yeast strain. Larger microcolonies, which are comprised of cells with a longer life span, are identified. Cells with increased life spans, isolated by any of these methods, are also part of the current invention.

The current invention also comprises methods of identifying agents which increase life span. Cells from a budding yeast strain with a known life span are exposed to the agent to be tested; the cells are then cultured and examined to determine whether they have longer life spans, using any of the methods described above. The presence of cells having longer life spans is indicative of the ability of the agent to increase life span of the cells.

In addition, the current invention pertains to genes which are involved in senescence of organisms, including yeast, bacteria and vertebrates, particularly mammals. Genes can be isolated by complementation analysis. For example, a genomic DNA library is constructed for the organism of interest, and is transformed into a mutant yeast strain having a mutated gene which contributes to longer life span, such as a mutant SIR4 gene. The DNA from the organism of interest is then isolated from those transformants which have the usual life span (i.e., those cells from the mutant yeast strain which no longer have a longer life span). Alternatively, genes which hybridize to a gene that is known to contribute to senescence, such as SIR4, can be isolated. The isolated genes, and the proteins encoded by the genes, are also the subject of the current invention. The subject invention also relates to DNA which encodes a protein which contributes to senscence in an organism (eukaryotes such as yeast and mammals, including humans, and prokaryotes). This includes UTH1

(SEQ ID No. 1), DNA which hybridizes to UTH1 and DNA which encodes the same amino acid sequence as that encoded by UTH1. Further, it includes DNA which hybridizes to SIR4 and DNA which encodes the same amino acid sequence as that encoded by SIR4. It also relates to mutant SIR4 DNA (which includes a stop at codon 1237), DNA which hybridizes to the mutant SIR4 DNA and DNA which encodes the same amino acid sequence as that encoded by mutant SIR4 DNA. The present invention also relates to proteins encoded by UTH1 DNA and the similar DNA sequences, as well as to proteins encoded by mutant SIR4 DNA and the similar mutant SIR4 DNA sequences.

Brief Description of the Figures

Figure 1 is a graphic representation of the mortality curves for two strains of *S. cerevisiae*, BWG1-7A (closed symbols), and PSY142 (open symbols).

Figure 2 is a graphic representation of the mean life spans of the four strains in the tetrad BKx1-14.

Figure 3 is a graphic representation of the viability of the tetrad strains after 7 days of starvation.

Figure 4 is a graphic representation of mortality curves for UTH1 mutants. Sample sizes were 37 cells (uth1-324, closed squares), 38 cells (uth1-328, open diamonds)), 38 cells (uth1-330, closed diamonds), 34 cells (uth1-342, closed squares with open centers), and 40 cells (14c, open squares with closed centers).

Figure 5 is a graphic representation of mortality curves for UTH2 mutants. Sample sizes were 40 cells (uth2-42, closed diamonds), and 40 cells (14c, open squares with closed centers).

Figure 6 is a graphic representation of mortality curves for UTH3 mutants. Sample sizes were 49 cells (uth3-26, closed diamonds), 40 cells (uth3-335, closed squares

with open centers), and 40 cells (14c, open squares with closed centers).

Figure 7 is a graphic representation of mortality curves for UTH4 mutants. Sample sizes were 40 cells (uth4-326, closed diamonds), and 40 cells (14c, open squares with closed centers).

Figure 8 is a graphic representation of the life span of haploid 14c (open squares) and diploid 14c (closed diamonds).

Figure 9 is a graphic representation of the life span of 14c (open squares), 14c with a disruption in the STE4 gene (closed diamonds), and 14c with a disruption in the STE12 gene (closed squares).

Figure 10 is a graphic representation of mortality curves for 14c (SIR4, open squares with closed centers), *sir4-42* (closed diamonds), and BKy104 (sir4, closed squares with open centers). Sample sizes were 139 cells (14c), 139 cells (*sir4-42*), and 136 cells (BKy104).

Figure 11 is a graphic representation of mortality curves for 14c (SIR4, open squares with closed centers), *sir4-42* (sir4, closed diamonds), and BKy109 (*sir4-42* + SIR4, closed squares with open centers). Sample sizes were 20 cells for all strains.

Figure 12 is a graphic representation of mortality curves for 14c (SIR4, open squares with closed centers), *sir4-42* (closed squares with open centers), and the isogenic deletion in sir1 derivatives (*sir4-42* Δsir1, open diamonds; SIR4 Δsir1, closed diamonds). Sample sizes were 20 cells (14c), 19 cells (SIR4 Δsir1), 18 cells (*sir4-42*), and 19 cells (*sir4-42* Δsir1).

Figure 13 is a graphic representation of mortality curves for 14c (SIR4, open squares with closed centers), *sir4-42* (closed squares with open centers), and the isogenic deletion in sir3 derivatives (*sir4-42* Δsir3, open diamonds; SIR4 Δsir3, closed diamonds). Sample sizes were

60 cells (14c), 20 cells (SIR4 Δsir1), 19 cells (*sir4-42*), and 30 cells (*sir4-42* Δsir1).

Figure 14 is a graphic representation of the mortality curves for 14c (SIR4, open squares) and SIR4 plus anti-SIR4 (closed diamonds). Sample sizes were 50 cells (14c) and 46 cells (SIR4 + Anti-SIR4).

Figure 15 is a depiction of the nucleic acid sequence (SEQ ID NO. 1), and the encoded amino acid sequence (SEQ ID NO. 2), of the UTH1 gene.

Detailed Description of the Invention

The present invention derives from the discovery that a particular gene is involved in senescence in yeast, and that a particular mutation in the gene causes an increase in life span of the yeast cells. As described below, Applicants have isolated longer-lived mutant yeast, in which the SIR4 gene has been mutated to generate a stop at codon 1237. As a result of this finding, it is now possible to isolate yeast cells with longer life spans, as well as to identify agents which contribute to longer life span. If is further possible to isolate genes involved in senescence, as well as the proteins encoded by these genes, and genes encoding proteins that contribute to longer life span. The following is a description of the discovery of a phenotype correlating with life span; the isolation of mutant yeast strains with longer life spans; the isolation and characterization of the mutant gene affecting life span; the requirements of other genes to lengthen life span; the effects of the mutant gene on telomeres; extension of life span expression of the carboxyl-terminus of the gene; a framework for relating silencing, aging, stress, and telomeres; methods of isolating strains with longer life spans; methods of identifying agents which affect life span; and methods of isolating genes involved in cellular senescence.

Identification of a Phenotype Correlating with Life Span

Because budding yeast cells divide asymmetrically into a large mother cell and a small daughter cell, the life span of any given mother cell in a particular colony can be measured. By visualizing growing cells in a microscope and micromanipulating away the daughter cell after each division, it is possible to follow a pedigree from each starting cell. The end of the life span for a given cell is indicated by a cessation of cell division. Life span is thus equated with the number of generations, or divisions, which give rise to daughter cells. The life span of a particular strain can be identified by the mean number of generations in several colonies. The chronological life span, therefore, is the approximate time necessary for one cell division, or for one generation to arise, multiplied by the number of divisions (generations) in the mean life span. A longer life span, as described herein, is measured as an increase in the mean life span of one strain as compared with the mean life span of a second strain.

To facilitate the identification of strains with altered life spans, a phenotype was sought which correlated with life span, yet which could be studied at the level of populations of cells (i.e., at a colony level). To this end, two parental strains were used, BWG1-7A (Guarente, L. et al., Cell 36:503-511 (1984)), and PSY142 (laboratory strain). These two strains had different mean life spans (18 generations for BWG1-7A, and 29 generations for PSY142), as shown in Figure 1. Four strains of *Saccharomyces cerevisiae* were generated by crossing the parental strains BWG1-7A and PSY142 and sporulating the diploid. These four segregants of this cross, known collectively as the tetrad BKx1-14 strains and individually as 14a, 14b, 14c, and 14d, have varying life spans (see Figure 2). When the tetrad strains were starved for nitrogen and carbon, it was discovered that starvation

contributed to cell death, and that the rate of cell death when starved was inversely proportional to the life span of the particular strain. That is, longer-lived strains were more resistant to starvation-induced death than shorter-lived strains (see Figure 3). Furthermore, strains with longer life spans yielded a greater recovery of viable cells after storage at 4°C for 4.5 months.

Isolation of Longer-lived Mutant Yeast Strains

To isolate longer-lived mutants, the shorter-lived strain 14c, which was relatively sensitive to starvation-induced cell death, was utilized. The yeast strain 14c has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852, USA, under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, on August 13, 1993; the accession number is 74236. All restrictions on the availability to the public of the deposited material will be irrevocably removed upon the granting of the patent. 14c yeast cells were mutagenized with ethylmethane sulfonate (EMS) (approximately 60% of cells killed); colonies were plated on supplemented minimal plates (yeast nitrogen base, 2% glucose, and those amino acids and nucleotides required for the strain) and replica-plated to plates lacking nitrogen and carbon (the starvation plates) (contents identical to supplemented minimal, without nitrogen and carbon). After incubation of the starvation plates at 30°C for five to ten days, the plates were replicated back to rich media plates (YPD) (1% yeast extract, 2% peptone, 2% dextrose). Most of the colonies consisted of dead cells, and thus did not grown on YPD; however, rare colonies contained living cells when plated back onto YPD (the "starvation resistant" colonies). Of 38,000 colonies, 39 were starvation resistant. Of these, eight had an extended life span

(extended 20-55%). To determine the life span, cells were taken from logarithmically growing liquid cultures and plated at low density on complete medium. The plates were incubated at 30°C for approximately three hours. At this time, daughter cells were isolated as buds that had emerged from mother cells, and moved with a Zeiss Micromanipulator to uninhabited regions of the plate. The life spans of these cells were determined by noting and removing all subsequent daughters they generated. The plates were incubated at 30°C during working hours and shifted to 4°C overnight. Life spans generated by this incubation schedule do not differ significantly from those generated by incubating cells continuously at 30°C (data not shown).

To determine whether the mutants were dominant or recessive, the eight starvation resistant mutants were crossed with an isogeneic derivative of 14c, BKy5, with the opposite mating type, sporulated, and shown to segregate 2:2 for stress-related phenotypes in more than 10 tetrads each. Genetic analysis indicated that seven were recessive and one was dominant. Complementation analysis showed that the recessive mutations fell into three genes (UTH 1, 2, and 3). The dominant mutation was not linked to representatives of any of these groups, and representatives of each group were not linked to each other. The dominant mutation was identified as a fourth gene (UTH4). Mortality curves for each complementation group (UTH 1-4) are shown in Figure 4 (UTH1), Figure 5 (UTH2), Figure 6 (UTH3), and Figure 7 (UTH4). The differences in life span were statistically significant by a Wilcoxen signed rank test.

Several different phenotypes were examined. To determine starvation resistance, haploid cells were grown in rich media to log phase, collected by centrifugation, and resuspended in minimal sporulation media for a period of seven to nine days. After starvation, cells were again

collected by centrifugation and plated on rich media to measure colony forming units (cfu)/ml. Colonies could be assayed for ability to withstand starvation by utilizing sporulation plates instead of liquid culture. Saturation density was measured by suspending logarithmically growing cells in rich medium liquid culture at a density of 10^6 cells/ml. Cultures were incubated for a period of five days with the number of cells/ml counted in a hemacytometer on a periodic basis. Control experiments indicated that the media was completely saturated after this time period. Heat shock resistance was determined by collecting logarithmically growing cells and plating them at a known concentration on rich media plates. The cells were heat-shocked at 55°C for periods varying from five minutes to one hour. Plates were then incubated at 40°C for three days and the number of colonies was counted. Growth on ethanol was measured by directly streaking a strain on either rich media containing ethanol or synthetic media supplemented with necessary nutrients and containing ethanol as the sole carbon source.

All eight mutants had phenotypes that were different from the parental 14c strain: better stress survival rate (resistance to nitrogen starvation); extended life span (as shown by more divisions); growth to a higher saturation density; heat shock resistance; enhanced growth on ethanol (a carbon source that induces the heat shock response in *S. cerevisiae*) (Plesset, Biochem. Biophys. Res. Comm. 108:1340-1345 (1982)); caffeine resistance; and paraquat sensitivity. In addition, one mutant, designated uth2-42, displayed two additional phenotypes: it mated poorly, and exhibited a pseudohyphal-like growth pattern. The latter phenotype has been observed in diploids that were starved for nitrogen (Gimeno, C. *et al.*, Cell 68:1077-1090 (1992)). Sterility and pseudohyphal-like growth both cosegregated

with stress tolerance. Moreover, in three complete tetrads it was found that a lengthened life span also cosegregated with the other mutant phenotypes.

Isolation and Characterization of Genes Affecting Life Span

Isolation of the UTH2 gene was conducted by the ability of UTH2 to restore mating to the uth2-42 strain, assayed by replica-plating transformants to a lawn of a tester strain of opposite mating type (CKy21). The uth2-42 mutant was transformed with a standard yeast genomic library, CT3, on a URA3 plasmid (Thompson, C., et al., Cell 73:1361-1375 (1993)), by standard methods (Guthrie, C. and G. Fink, Methods in Enzymology, 1991), and Ura+ colonies which were resistant to paraquat were selected. Transformed colonies were tested for their ability to complement the mating detect in the uth2-42 mutant. Plates containing library-transformed colonies were replica-plated onto permissive plates containing a lawn of strain CKy21. Cells were incubated at room temperature for one day to allow mating and then were replica-plated to plates selective for diploid growth. Colonies were picked which clearly grew on the selective plates. Plasmids were recovered from these colonies by standard methods and re-transformed into uth2-42 mutant cells. One plasmid restored the mating efficiency of the uth2-42 mutant. This plasmid, pBK40, also conferred heat shock sensitivity and starvation sensitivity to uth2-42, making it a good candidate for the UTH2 gene. pBK40 contained an insert of about 8 kb.

A 1.6 kb fragment located entirely within the pBK40 library insert was random primed by manufacturer's protocol (U.S. Biochemical), and used to probe a panel of lambda clones containing yeast DNA ((Riles, L. et al., Genetics 134:81-150 (1993)). Only one clone, the lambda clone that

hybridized contained SIR4, showed a distinguishable signal.

SIR4 is a component of the yeast silencing complex that represses copies of MATα and MATa information and HML and HMR (Hartwell, L.H. J. Cell. Biol. 85:811-822 (1980); Laurenson, P. and J. Rine, Microbiol. Rev. 56:543-560 (1992); Rine, J. and I. Herskowitz, Genetics 116:9-22 (1987)). Restriction mapping of pBK40 indicated that it contained SIR4 and at least 1 kb of flanking DNA to either side. To determine linkage, the insert was transferred to a LEU2-containing integrating vector and targeted to the SIR4 locus in BKy5. This integrant (BKy30) was mated with uth2-42 (containing pBK40 to allow mating), and after eviction of pBK40, the diploid sporulated. Thirteen of thirteen tetrads contained 2 Leu+, fertile:2 Leu-, sterile segregants, showing that SIR4 is tightly linked to the uth2-42 mutation. It was concluded that UTH2 was SIR4; therefore, uth2-42 was designated *sir4-42*.

The SIR4 gene is one of a series of genes (SIR1-4) involved in mating type switching. The SIR1-4 genes silence reserve copies of a and α information at the HML and HMR loci which are located to the left and right of the MAT mating type locus (see Rine, J. and Herskowitz, I., Genetics 116:9-22 (1987), for overview). The SIR1-4 genes also silence genes located at the telomeres of yeast chromosomes (Aparicio, O. M. et al., Cell 66(6):1279-1287 (1991)). No other functions had previously been attributed to these genes.

The SIR4 mutant is sterile because it expresses a and α information simultaneously. The effect of the SIR4 deletion was not simply because cells simultaneously expressed a and α information: the isogeneic diploid of 14c, BKy6, did not live longer than the haploid parents (14c and BKy5) (see Figure 8). To generate BKy5, strain 14c was transformed with a (GAL-HO) plasmid and plated on

galactose medium to induce mating type switching (Guthrie, C. and G. Fink, Methods in Enzymology, 1991). Colonies were tested by mating to CKy20 or CKy21 to determine their mating type; a MATa colony was picked and the GAL-HO plasmid was segregated using 5-FOA (Boeke, J.D. *et al.*, Meth. Enzymol. 154:164-175 (1987)). This strain, BKy5, was mated to 14c and zygotes were isolated by micromanipulation to generate BKy6. To verify that BKy6 was a diploid, the strain was shown to be sporulation-competent.

Further, sterility *per se* was not the cause of the longer life span. Disrupting STE4 or STE12, genes involved in aspects of mating different than those of SIR4, did not affect life span (see Figure 9). The disruption of STE4 was constructed using as described (Whiteway, M. *et al.*. cell 56:467-477 (1989)).

In addition, introduction of a plasmid which expressed MATα into BKy5 did not lengthen life span. The effects of sterility on life span are shown in Table 1, below. The maximum life span indicates the number of daughters produced by the oldest mother cell.

Table 1: The Effects of Sterility on Mean Life Span

Strain	Sample Size	Mean Life Span	Maximum Life Span
BKy1-14c	20	15.6	25
BKy5	20	14.5	25
BKy6	20	15.3	27
BKy100 (ste4Δ)	20	15.9	24
BKy101 (ste12Δ)	20	16.5	24
BKy5 + Matα	20	14.6	26

Because the stress and mating phenotypes of *sir4-42* were recessive, it was surmised that the phenotype of a SIR4 null mutation would mimic that of *sir4-42*. The entire

SIR4 gene was deleted in 14c: the region from 153 base pairs 5′ to SIR4 through the entire open reading frame was deleted and replaced with the URA3 gene using the plasmid pAR59 provided by J. Broach (Marshall, M. *et al.*, Mol. Cell. Biol. 7:4441-4452 (1987)). The sir4 deletion was confirmed by southern analysis. The resultant deleted strain, BKy104, was indeed stress tolerant and sterile (data not shown). Importantly, however, it did not have a lengthened life span; in fact, the deletion shortened life span by a small, but statistically significant, degree (see Figure 10).

These data suggested that the effect of *sir4-42* on life span, unlike its effects on stress and mating, might be due to a gain of function. To test this, it was investigated whether the sir-42 allele was dominant to SIR4 for the phenotype of lengthened life span. The wild type SIR4 was transferred to an integrating vector and targeted to URA3 in the *sir4-42* mutant. The integration plasmids were generated by subcloning the entire library insert containing SIR4 from pBK40 into pRS305 or pRS306 by a NotI SalI double digest (Sikorski, R.S. and P. Hieter, Genetics 122:19-27 (1989)). Integration was directed to the URA3 locus by a StuI digest, and was verified by Southern analysis. The resulting SIR4-*sir4-42* haploid (BKy109) was stress sensitive and mated efficiently, as expected. However, the life span of this strain was intermediate between the SIR4 parent, 14c, and the *sir4-42* mutant, as shown in Figure 11. Statistical analysis determined that the mean life span of BKy109 was significantly different from the means of both *sir4-42* and 14c. The *sir4-42* mutation therefore is semi-dominant with respect to life span.

As a second test for dominance mating was used to construct isogenic diploids, SIR4/SIR4 (BKy6), SIR4/*sir4-42* (BKy17), and *sir4-42*/*sir4-42* (BKy28) (using the SIR4

plasmid, pBK40, to permit mating in *sir4-42* mutants). BKy19 was generating by mating the *sir4-42* mutant containing pBK40 to 14c and subsequently removing the plasmid with 5-FOA. BKy17 was sporulated and a MATa *sir4-42* segregant (BKy21) was used to generate the homozygous *sir4-42* diploid (BKy28). BKy21 carrying pBK40 was mated to the *sir4-42* mutant also carrying pBK40 and diploids were isolated. The homozygous diploids have life spans similar to their haploid parents, and the heterozygous diploid displayed a life span intermediate between the homozygotes (data not shown). these findings clearly show that the extended life span in the *sir4-42* mutant is semi-dominant, and therefore, due to a gain of function mutation.

Gap repair was utilized to clone both the wild type SIR4 allele from 14c and the *sir4-42* allele from the SIR4 mutant strain (Guthrie, C. and G. Fink, Methods in Enzymology, 1991). A SmaI AatII double digest was performed to remove the coding region of SIR4 from pBK40. The linear plasmid was gel purified and transformed into either 14c or the *sir4-42* mutant. Ura+ colonies were picked and the plasmids were recovered by standard methods. Restriction digests were conducted to determine if the gap repair event was successful. To localize the mutation within SIR4, digests were conducted with AatII, SmaI, and SphI, all of which have one site in the SIR4 gene and another within the pBK40 insert, either 5′ or 3′ to SIR4. These linearized plasmids were transformed into *sir4-42* and transformants were tested for their ability to complement the *sir4-42*-associated mating defect. This analysis localized the mutation to the region spanning codons 743 to the UAA stop at the end of the 1358 residue SIR4 open reading frame. The clone was shown to contain the mutation by a functional test in which it was transferred to an integrating vector, and targeted to LEU2 in strain BKy104 (Δsir4). Integration was directed to the LEU2 locus by a

XcmI digest, and verified by Southern analysis. The resulting strain had an extended life span, indicating that the integrating vector contained the *sir4-42* allele (data not shown). The SmaI fragments from the mutant or wild type SIR4 gene, which contained the region spanning 743 to the UAA stop at the end of the 1358 residue SIR4 open reading frame, were subcloned into Bluescript (Stratagene). Sequencing primers were made approximately 200 base pairs apart for this entire region, and it was sequenced by the single-strand approach (Sequenase version 2, U.S. Biochemicals). A single difference was found in the mutant which generated a stop at codon 1237, removing 121 residues from the SIR4 gene product.

A second gene involved in senescence in yeast, corresponding to UTH1 described above, has been isolated and sequenced. The UTH1 mutation, described above, rendered 14c sensitive to paraquat. The UTH1 gene was cloned from the CT3 library by its ability to confer resistance to paraquat. The sequence was obtained using standard methods. The nucleic acid sequence (SEQ ID NO. 1), and the encoded amino acid sequence (SEQ ID NO. 2), are shown in Figure 15.

The Lengthening of Life Span by *sir4-42* Requires SIR3

It was investigated whether *sir4-42* acted alone or in concert with other members of the SIR complex. The activities of SIR2, SIR3, and SIR4 are closely coupled in that all are required for silencing at the HM loci and at telomeres (Aparicio, O. M. et al., Cell 66(6):1279-1287 (1991); Rine, J. and Herskowitz, I., Genetics 116:9-22 (1987)). The function of SIR1 is different in that it is only required at the HM loci (Aparicio, O. M. et al., Cell 66(6):1279-1287 (1991)), and even there, its requirement is not absolute (Pillus, L. and J. Rine, Cell 59:637-647 (1989)). To determine whether SIR3 and SIR1 were required

for the extension of life span, the genes were disrupted in the *sir4-42* mutant, and, as a control, in 14c. The sir1 deletion was generated using plasmid pJI23.2 which removes the C-terminal 335 amino acids from the 648 amino acid protein (Ivy, J.M. *et al.*, Mol. Cell.Biol. 6:688-702 (1986)). The sir3 deletion was constructed by deleting 123 amino acids at the C-terminus of SIR3. The sir1 disruptions did not exert any effect on the *sir4-42* mutant or its SIR4 parent (Figure 12). In contrast, the sir3 disruption abolished the extension of life span conferred by *sir4-42* (Figure 13). This shortening of life span in the *sir4-42* strain was specific because disruption of SIR3 did not alter the life span of the SIR4 patent (Figure 13). Thus, the gain of function caused by *sir4-42* appears to be an activity of the entire SIR complex, and not SIR4 alone.

Effects of the *sir4-42* Mutation on Telomeres

Because the *sir4-42* mutation results in a loss of activity at HM loci, it is possible that the mutation redirects the SIR complex to another chromosomal location, resulting in the observed extension in life span. One obvious possible location was telomeres, because loss of function mutations in SIR2, SIR3, or SIR4 relieve silencing at telomeres and also result in shorter telomeres (Aparicio, O. M. et al., Cell 66(6):1279-1287 (1991); Palladino, F. *et al.*, Cell 75:543-555 (1993)). In mammalian cells, telomeres have been shown to shorten with age (Harley, C.B. *et al.*, Nature 345:458-460 (1990)), and this shortening has been proposed as a causative agent of aging (Allsopp, R.C. *et al.*, PNAS, USA 89:10114-10118 (1992); Olovnikov, A.M. J. Theor. Biol. 41:181-190 (1973)). If telomere shortening imposed a limit to life span, then excessive recruitment of SIR complex might counter aging by lengthening telomeres. Therefore, the length of telomeres

in 14c and its Δsir4 and *sir4-42* mutant derivatives was determined. Total genomic DNA was isolated, digested with XhoI, and separated on a 0.7% agarose gel and transferred to a GeneScreen Plus Hybridization Transfer Membrane (NEN Research Products). Hybridization and wash conditions were as suggested by the manufacturer. A plasmid containing 600 base pairs located within the conserved Y' region of yeast telomeres, supplied by V. Zakian, was nick translated (GIBCO BRL) and used as a probe (Chan, C.S.M. and B.K. Tye, Cell 33:563-573 (1983)). This probe overlapped the XhoI site and thus hybridized to fragments both telomere-proximal and telomere-distal to the restriction site. Most yeast telomeres contain the Y' region (Walmsley, R.M. *et al.*, Nature 310:157-160 (1984)). Deletion of SIR4 resulted in a shortening of telomeres by approximately 50-100 bases (Palladino, F. *et al.*, Cell 75:543-555 (1993)). Surprisingly, the length of telomeres in the *sir4-42* mutant was indistinguishable from the Δsir4 mutant, indicating that the mutant behaved like the deletion with respect to activity at telomeres. Separate experiments confirmed that silencing at telomeres was also alleviated in the *sir4-42* mutant just as in the Δsir4 strain (data not shown). Thus, the *sir4-42* exhibits a loss of function phenotype. However, because *sir4-42* extends life span and Δsir4 does not, the lengthened life span is probably unrelated to telomere length or silencing.

Expression of the Carboxyl-terminus of SIR4 Extends Life Span

Since the *sir4-42* mutation removes the carboxyl-terminus of the protein, it is possible that this fragment of SIR4 localized the complex to HM loci and telomeres. Thus, overexpression of a carboxyl-terminal fragment of SIR4 might compete with the wild type protein for recruitment to HM loci and telomeres. A construct

expressing only the carboxyl 154 residues of SIR4 has been shown to behave as an anti-SIR4 dominant negative mutant with respect to silencing at HM loci (Ivy, J.M. *et al.*, Mol. Cell.Biol. 6:688-702 (1986); Marshall, M. *et al.*, Mol. Cell. Biol. 7:4441-4452 (1987)). Therefore, a construct that expresses the carboxyl-terminal region of SIR4 (Ivy, J. et al., Mol. Cell Biol. 6:688-702 (1986)) was used to antagonize the native SIR4 protein and render cells sir4-. Transformation of this construct into 14c confirmed that it functioned as a dominant negative inhibitor of mating. The transformant was also stress resistant, as expected. Strikingly, the construct also extended the life span by about 30% (see Figure 14). The strain labeled SIR4 + Anti-SIR4 is 14c transformed with the plasmid pJH3A, a 2 μ plasmid containing the C-terminal 154 amino acids of the SIR4 gene (Ivy, J. et al., Mol. Cell Biol. 6:688-702 (1986)).

Summary of Yeast Strains Described Above

Table 2 depicts the strain and genotype of all yeast strains described herein. All strains were generated in this study except BWG1-7A which is described in Guarente, L. and T. Mason, Cell 32:1279-1286 (1983)), and the mating testers CKy20 and CKy21 which were gifts of C. Kaiser. The terminology LEU2/*sir4-42* in the strain BKy107 means the *sir4-42* allele has been integrated at the LEU2 locus, for example.

Table 2 Yeast Strains Used in this Study

Strain	Genotype
BWG1-7A	Mata ade1-100 his4-519leu2-3,2-112 ura3-52
PSY142	Matα leu2-3,2-112lys2-801 ura3-52
BKy1	Mata ade1-100 his4-519 leu2-3,2-112 LYS2 ura3-52 Matα ADE HIS4 leu2-3,2-112 lys2-801 ura3-52
BKy1-14a	Mata ade1-100 leu2-3,2-112 lys2-801 ura-3-52
BKy1-14b	Matα leu2-3,2-112 ura3-52
BKy1-14c	Matα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52
BKy1-14d	Mata his4-519 leu2-3,2-112 ura3-52
BKy5	Mata ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52
BKy6	Mata ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 Matα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52
BKy17	Mata ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 SIR4 Matα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42
BKy21	Mata ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42
BKy28	Mata ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42 Matα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42
BKy30	Mata ade1-100 his4-519 leu2-3,2-112 lys2-801 ura 3-52 SIR4/LEU2
Bky100	Matα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 Ste4::URA3
BKy101	Matα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 ste12::URA3

Table 2, continued

BKy102	Matα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir1::LEU2
BKy103	Matα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir3::URA3
BKy104	Matα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4::URA3
BKY105	Matα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42 sir1::LEU2
BKy106	Matα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42 sir3::URA3
Bky107	Matα ade1-100 his4-519 lys2-801 ura3-52 sir4::URA3 LEU2/sir4-42
BKy108	Matα ade1-100 his4-519 leu2-3,2-112 ly2-801 sir4-42 URA3/SIR4
CKy20	Matα arg1 tsm11
CKy21	Mata arg1 tsm11

Framework for Relating Silencing, Aging, Stress, and Telomeres

Table 3 summarizes the effects of three mutant alleles of SIR4 that alleviate silencing and also promote stress resistance.

Table 3 Phenotypes of SIR4 Alleles

Allele	Amino Acids	Mating	Stress Resistance	% Life Span Increase
SIR4	1-1358	+	Sensitive	---
sir4-42	1-1237	-	Resistant	30-60%
sir4Δ	---	-	Resistant	none
SIR4 + Anti-SIR4	1-1358 + 1205-1358	-	Resistant	20-45%

Deletion of SIR3 has effects indistinguishable from deletion of SIR4 (data not shown). Of all of these mutations, however, only *sir4-42* extends life span. To explain these findings, it is proposed that a locus that is repressed by the SIR complex can promote resistance to stress when repression is eliminated. In principle, this locus could be linked to HML, HMR, a telomere, or reside at some other location. Linkage to HM loci is not plausible, however, because deletion of SIR1, which weakens repression at the HM loci, does not promote stress resistance. For simplicity, it is suggested that there is a telomere-linked, stress-resistant locus under SIR control.

It is further suggested that the lengthening of life span is due to a different locus, termed AGE, that is independent of effects ad HM loci or telomeres. The repression of the "AGE" locus by SIR4 is essential to longevity, according to this view, and aging may result from a breakdown in the silencing of that locus. It is, of course, possible that silencing at more than one chromosomal regions governs aging. In any case, the "AGE"

locus is proposed to be unlinked to telomeres or HM loci because both the *sir4-42* mutation and the Δsir4 eliminate silencing at HM loci and at telomeres, but only the *sir4-42* allele extends life span. Further, the extension of life span by *sir4-42* is semi-dominant in a strain also containing SIR4, indicating that it is a gain of function mutation with regard to life span. The function gained in the mutant must relate to the normal silencing activity of the SIR complex because the ability of *sir4-42* to promote longevity requires the integrity of SIR3.

It is also suggested that the *sir4-42* mutation prevents recruitment of the SIR complex to HML, HMR, and telomeres, rendering the complex more available for any other site of action in the cell. The carboxyl 121 residues that are missing in the *sir4-42* mutant may be important in the recruitment of the SIR complex to these chromosomal sites. Consistent with the view that the carboxyl terminus of SIR4 helps localize the SIRs to HM loci and telomeres, overexpression of the carboxyl 163 residues of SIR4 is known to exert a dominant negative effect on repression at HM loci (Ivy, J. et al., Mol. Cell Biol. 6:688-702 (1986); Marshall, M. *et al.*, Mol. Cell. Biol. 7:4441-4452 (1987)). Expression of this SIR4 fragment, in addition to blocking repression at HML and HMR, promoted longevity.

A breakdown in silencing by the SIR complex may be causally related to aging in *S. cerevisiae*. The identification of SIR4 as a gene that affects life span in yeast thus appears to relate telomeres and aging. However, as described above, telomeres in the *sir4-42* strain, just as in the Δsir4 null mutant, are shorter than wild type. This suggests that telomere length is not causally related to aging. Nevertheless, it is theoretically possible that the mutation counters telomere shortening selectively in old cells.

Methods of Isolating Strains with Increased Life Span

The techniques described above can be used to isolate other yeast strains with increased life spans, and thereby to isolate other genes, from yeast and other cell types (e.g. vertebrate, mammalian) involved in senescence. Any budding yeast strain for which the life span is known can be utilized. The life span of the strain can be determined by calculating the mean number of generations before senescence in a sample of colonies of the strain of interest. A sample of the strain of interest is exposed to a mutagen, such as ethylmethane sulfonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), or ultraviolet irradiation. Mutants with increase life spans can then be isolated as follows.

Starvation-resistance method Yeast cells that have been exposed to mutagen are plated with minimal nutrients (including carbon and nitrogen sources, as well as the amino acids and nucleotides that are required by the particular strain for growth). The minimal plates are replica-plated to plates lacking vital nutrients, such as nitrogen and carbon (the starvation plates). After incubation of the starvation plates at a temperature appropriate for growth, for several days, the starvation plates are replicated back to rich media plates. The rare colonies containing living cells when plated back onto rich medium (the "starvation resistant" colonies) are then examined to determine whether the life span is extended. Life span is calculated as described above. This method is particularly appropriate for short-lived strains, which are more sensitive to starvation.

Cell surface labelling method This method takes advantage of the fact that the cell surface (including the cell membrane and cell wall) of a daughter cell in some budding yeast, such as *S. cerevisiae*, is fabricated entirely of new materials: when the cell surface of the

mother cell is labelled, the surface of the daughter cells remains unlabelled. In one embodiment, the cell surface is labelled with biotin. When avidin linked to fluorescence is coupled to the biotin, the cell surface fluoresces. Alternatively, any other method of labelling the cell surface with a fluorescent marker is appropriate. Daughter cells remain unlabelled (will not fluoresce). Fluorescently labelled yeast cells are plated and cultured for a period of time greater than the life span of the non-mutant strain (as measured by time necessary for one cell division, multiplied by the number of divisions, or generations, in the life span). If desired, the yeast cells may be sampled at regular time intervals in order to monitor the plating efficiency of the cells; the efficiency will drop precipitously after the chronological life span has passed. The yeast cells are then subjected to *fluorescence-activated cell sorting (FACS)*, to isolate the fluorescently labelled cells. The fluorescent cells are then replated; only mutants with increased life spans will grow.

Temperature-sensitive method A temperature-sensitive mutant strain, in which the daughter cells die at the non-permissive temperature, is utilized. For example, yeast cells with a mutation in the *mdm2-2* gene (also known as the ole-1 gene) (McConnell, S. et al., J. Cell Biol. 111:967-976 (1990)) bud forth living daughter cells at 30°C, but not at 37°C, because of a failure in appropriate organelle segregation at the higher temperature (mitochondria are not put into daughter cells). In such a temperature-sensitive mutant, the daughter cells bud off from the mother cell and die at the non-permissive temperature; the dead daughter cells remain near the mother cell. Therefore, each mother cell grown at the non-permissive temperature generates a microcolony of N cells, where N is equal to the number of generations in the life span of the mother cell. Mutant

strains will display microcolonies wherein the number of cells is greater than N.

To isolate mutants, cells are plated at the permissive temperature. A sample of cells from each colony is then transferred to a plate to be grown at the non-permissive temperature. Microcolonies with cell number greater than N are indicative of mutants; cells from the colonies which have been identified as mutant can be selected from the plates grown at the permissive temperature. Alternatively, cells are plated directly at the non-permissive temperature, and grown for a period of time greater than the life span as measured by time necessary for one cell division, multiplied by the number of divisions, or generations, in the life span. If desired, the yeast cells may be sampled at regular time intervals in order to monitor the plating efficiency of the cells; the efficiency will drop precipitously after the chronological life span has passed. After this time, the plates are shifted back to the permissive temperature. Only longer-lived mutants will grow after the temperature shift.

Methods of Identifying Agents Which Affect Life Span

The above-described methods for isolating mutant yeast cells with a longer life span can be employed to identify agents which alter the life span of a yeast strain. In this embodiment of the current invention, the yeast strain of interest, for which the life span is known or has been calculated, is exposed to the agent to be tested rather than subjected to a mutagen. The samples thus exposed are then examined for longer-lived colonies, using any of the methods described above. Colonies exhibiting a longer life span in the presence of the agent than in the absence of the agent are indicative of the ability of the agent to increase life span, or to postpone senescence. Agents include drugs, peptides, oligonucleotides, and genes

encoding proteins that increase life span, such as genes isolated by the methods described below.

Methods of Isolating Genes Involved in Altering Life Span

Genes which contribute to senescence can be isolated by complementation analysis, or by isolation of DNA homologous to other genes known to contribute to senescence. In one embodiment of the current invention, cells from a budding yeast strain, such as 14c, in which the SIR4 gene has been mutated as described above, and which as a result have a longer life span, are utilized. The SIR4 gene can be mutated through site-specific mutagenesis, for example. A genomic DNA library generated from an organism of interest, including another yeast strain, bacteria, or mammals, is used to transform the yeast cells. The cells are then plated and grown. Those yeast cells which exhibit the usual life span of the yeast strain, rather than the longer life of the cells in which SIR4 is mutated, are selected. These cells contain DNA from the organism of interest which comprises a gene that contributes to senescence. The DNA from the organism of interest is then isolated from these yeast cells.

Genes which contribute to longer life span can also be isolated by complementation analysis, or by isolation of DNA homologous to other genes known to contribute to longer life span. In one embodiment of the current invention, cells from a budding yeast strain, such as 14c, are utilized. These cells should have a normal life span; i.e., the SIR4 gene should not be mutated. A genomic DNA library generated from an organism of interest, including another yeast strain, bacteria, or mammals, is used to transform the yeast cells. The cells are then plated and grown. Those yeast cells which exhibit a longer life span of the yeast strain, rather than the usual life span of the cells, are selected. These cells contain DNA from the

organism of interest which comprises a gene that contributes to longer life span (i.e., a gene that increases life span). The DNA from the organism of interest is then isolated from these yeast cells. In another embodiment, genes in other organisms that are the functional equivalent of SIR4 in yeast can be investigated to determine whether a mutation corresponding to the SIR4 mutation (stop at codon 1237) results in a mutated gene that contributes to longer life span.

In another embodiment of the current invention, homologous genes can be isolated by hybridization. In one particular embodiment, a labelled DNA fragment comprising the SIR4 gene or the UTH1 gene is used to probe cellular DNA from an organism of interest under high, medium or low hybridization stringency conditions, depending on the degree of homology sought. For description of appropriate stringency conditions, see Sambrook *et al.*, eds., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, 1989, or Ausubel, F.M. et al., eds. Current Protocols in Molecular Biology, 1994. DNA hybridizing to the probe is isolated, and complementation analysis is performed to verify that the DNA comprises a gene which contributes to senescence. In one embodiment, DNA from an organism of interest is hybridized under high stringency conditions to DNA comprising a mutated SIR4 gene (i.e., a stop at codon 1237). Alternatively, labelled DNA comprising genes isolated by the complementation method described above can be used as the probe.

Homologous genes can also be found by the polymerase chain reaction (PCR) (see Sakai, R. K. et al., Science 230:1350-4 (1985), and Sakai, R. K. et al., Science 239: 487-91 (1988)). Synthetic oligonucleotide primers which comprise regions of the SIR4 gene or the UTH1 gene can be used. In one embodiment, synthetic oligonucleotide primers which comprise the region of the SIR4 gene that contains

the mutation (the stop at codon 1237) are used. Alternatively, oligonucleotides can be patterned after any gene, such as those isolated by this method or any of the above methods, which contributes to senescence or to longer life span. The oligonucleotides are utilized in PCR to generate multiple copies of DNA of interest from a sample of genomic DNA from the organism of interest. The DNA multiplied in PCR is then isolated, and complementation analysis is performed to verify that the DNA comprises a functional gene which contributes to senescence or to longer life span. Once genes have been isolated using these methods, standard procedures can then be used to isolate the proteins encoded by the genes.

Methods of Increasing Life Span in Yeast

Because the *sir4-42* mutation is a semi-dominant mutation, and because addition of "anti-SIR4" (residues 1205-1358 of SIR4) to yeast cells increases the life span by 20-45%, it is now possible to increase the life span of any cell by adding "anti-SIR4". For example, a plasmid which expresses residues 1205-1358 can be inserted into the cell of interest. Expression of the anti-SIR4 protein will increase the life span. The life span can also be increased by adding mutant SIR4 protein (protein produced by the mutated SIR4 gene, in which there is the stop at codon 1237). For example, a plasmid which expresses the mutant SIR4 protein can be inserted into the cell of interest. Alternatively, "anti-SIR4" protein or protein produced by the mutant SIR4 gene can be added to the cell, thereby increasing the cell's life span.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many

equivalents to specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

The invention claimed is:

1. A method of isolating mutant yeast cells with an increased life span, comprising the steps of:
   a) exposing a sample of yeast cells from a budding yeast strain, for which the life span is known, to a mutagen;
   b) plating the sample of cells on minimal medium necessary for growth of yeast cells, thereby generating an original plate;
   c) replica-plating the original plate to a plate with a medium lacking nutrients necessary for growth of yeast cells, thereby generating a replica plate;
   d) culturing the original plate and the replica plate under conditions appropriate for growth of yeast cells;
   e) replica-plating the replica plate to enriched medium, thereby generating an enriched plate;
   f) culturing the enriched plates under conditions for growth of yeast cells, thereby generating colonies of yeast cells which are starvation resistant;
   g) calculating the life span of yeast cells in the starvation resistant colonies; and
   h) selecting those yeast cells with a life span that is longer than the known life span of the yeast strain.

2. A method of isolating mutant yeast cells with an increased life span, comprising the steps of:
   a) exposing a sample of yeast cells from a budding yeast strain, for which the life span is known, to a mutagen;

b) labelling the cell surface of the yeast cells with a fluorescent marker, thereby generating fluorescent yeast cells;

c) culturing the yeast cells under conditions for growth of yeast cells, and for a period of time greater than the chronological life span of the strain;

d) subjecting the yeast cells to fluorescence-activated cell sorting, thereby separating fluorescent yeast cells from non-fluorescent yeast cells;

e) replating the fluorescent yeast cells, under conditions for growth of yeast cells;

wherein those fluorescent yeast cells which grow after replating are mutant yeast cells having an increased life span.

3. A method of isolating mutant yeast cells with an increased life span, comprising the steps of:

a) exposing a sample of yeast cells from a temperature-sensitive budding yeast strain, in which the daughter cells die at the nonpermissive temperature, and for which the life span is known, to a mutagen;

b) plating the yeast cells, and cultivating the yeast cells at the permissive temperature and under conditions for growth of yeast cells;

c) transferring a sample of yeast cells from each colony of the plate at the permissive temperature to a second plate;

d) cultivating the yeast cells transferred to the second plate at the nonpermissive temperature, thereby generating microcolonies of yeast cells; and

e) calculating the number of yeast cells in the microcolonies,

wherein if a microcolony consists of a number of yeast cells that is greater than the number of generations in the life span of the yeast strain, the microcolony contains mutant yeast cells having an increased life span.

4. A method of isolating mutant yeast cells with an increased life span, comprising the steps of:

a) exposing a sample of yeast cells from a temperature-sensitive budding yeast strain, in which the daughter cells die at the nonpermissive temperature, and for which the life span is known, to a mutagen;

b) plating the yeast cells, and cultivating the yeast cells at the nonpermissive temperature for a period of time greater than the chronological life span of the strain, thereby generating microcolonies of yeast cells; and

c) shifting the microcolonies to the permissive temperature,

wherein those yeast cells which grow after the shift to the permissive temperature are mutant yeast cells having an increased life span.

5. The method of any one of Claims 1 to 4, wherein the yeast is *Saccharomyces cerevisiae*.

6. The method of any one of Claims 1 to 5, wherein the yeast strain is 14c.

7. A mutant yeast cell with an increased life span, isolated by any one of the methods of Claims 1 to 6.

8. The method of either Claim 3 or Claim 4, wherein the temperature sensitive budding yeast strain is *mdm2-2*.

9. A method of identifying an agent which increases the life span of yeast cells, comprising the steps of:

a) exposing a sample of yeast cells from a budding yeast strain, for which the life span is known, to the agent to be tested;

b) plating the sample of yeast cells with the minimal medium necessary for growth of yeast cells, thereby generating an original plate;

c) replica-plating the original plate to a plate with a medium lacking nutrients necessary for growth of yeast cells, thereby generating a replica plate;

d) culturing the original plate and the replica plate under conditions appropriate for growth of yeast cells;

e) replica-plating the replica plate to an enriched medium, thereby generating an enriched plate;

f) culturing the enriched plates under conditions for growth of yeast cells; and

g) calculating the life span of yeast cells which grow on enriched plates,

wherein the presence of yeast cells with a longer life span than that of the known life span of the yeast strain is indicative of the ability of the agent to increase life span.

10. A method of identifying an agent which increases the life span of yeast cells, comprising the steps of:

a) exposing a sample of yeast cells from a budding yeast strain, for which the life span is known, to the agent to be tested;

b) labelling the cell surface of the yeast cells with a fluorescent marker, thereby generating fluorescent yeast cells;

c) culturing the yeast cells under conditions for growth of yeast cells, and for a period of time greater than the chronological life span of the strain;

d) subjecting the yeast cells to fluorescence-activated cell sorting, thereby separating fluorescent yeast cells from non-fluorescent yeast cells;

e) replating the fluorescent yeast cells, under conditions for growth of yeast cells;

wherein growth of fluorescent yeast cells after replating is indicative of the capability of the agent to increase life span.

11. A method of identifying an agent which increases the life span of yeast cells, comprising the steps of:

a) exposing a sample of yeast cells from a temperature-sensitive budding yeast strain, in which the daughter cells die at the nonpermissive temperature, and for which the life span is known, to the agent to be tested;

b) plating the yeast cells, and cultivating the yeast cells at the permissive temperature and under conditions for growth of yeast cells;

c) transferring a sample of yeast cells from each colony of the plate at the permissive temperature to a second plate;

d) cultivating the yeast cells transferred to the second plate at the nonpermissive temperature, thereby generating microcolonies of yeast cells; and

e) calculating the number of yeast cells in the microcolonies,

wherein the existence of microcolonies consisting of a number of yeast cells that is greater than the number of generations in the life span of the yeast strain, is indicative of the capability of the agent to increase life span.

12. A method of identifying an agent which increases the life span of yeast cells, comprising the steps of:

a) exposing a sample of yeast cells from a temperature-sensitive budding yeast strain, in which the daughter cells die at the nonpermissive temperature, and for which the life span is known, to the agent to be tested;

b) plating the yeast cells, and cultivating the yeast cells at the nonpermissive temperature for a period of time greater than the chronological life span of the strain, thereby generating microcolonies of yeast cells; and

c) shifting the microcolonies to the permissive temperature,

wherein the growth of yeast cells after the shift to the permissive temperature is indicative of the capability of the agent to increase life span.

13. The method of any one of Claims 9 to 12, wherein the yeast is *Saccharomyces cerevisiae*.

14. The method of any one of Claims 9 to 13, wherein the yeast strain is 14c.

15. An agent which increases the life span of yeast cells, isolated by any one of the methods of Claims 9 to 14.

16. The method of either Claim 11 or Claim 12, wherein the temperature sensitive budding yeast strain is *mdm2-2*.

17. The method of any one of Claims 9 to 16, wherein the agent is a gene encoding a protein that increases life span.

18. A method of isolating a gene which encodes a protein that contributes to senescence in an organism, comprising the steps of:

a) generating a genomic DNA library from the organism of interest;

b) transforming yeast cells from a budding yeast strain, in which the SIR4 gene has been mutated to generate a stop at codon 1237, with the library;

c) plating the transformed yeast cells, and culturing them under conditions for growth of yeast cells;

d) calculating the life span of the colonies of transformed yeast cells;

e) selecting yeast cells from colonies in which the life span of the yeast cells is approximately equal to the life span of the yeast strain in which the SIR4 gene has not been mutated to generate a stop at codon 1237, thereby obtaining yeast cells containing senescence genes from the organism of interest; and

f) isolating from the selected yeast cells the DNA from the organism of interest,

thereby obtaining a gene encoding a protein that contributes to senescence.

19. The method of Claim 18, wherein the budding yeast is *Saccharomyces cerevisiae*.

20. The method of Claim 19, wherein the yeast strain is 14c.

21. A gene which encodes a protein that contributes to senescence in an organism, isolated by the method of any one of Claims 18-20.

22. A gene which encodes a protein that contributes to senescence in an organism, and which hybridizes under conditions of medium stringency to a gene isolated by the method of any one of Claims 18-20.

23. A protein encoded by the gene of either of Claim 21 or Claim 22.

24. A method of isolating DNA which is homologous to a gene contributing to senescence in an organism, comprising the steps of:

a) generating a genomic DNA library from the organism of interest;

b) contacting the library with a labeled probe comprising DNA encoding the SIR4 gene or the UTH1 gene, under conditions of low stringency; and

c) isolating from the library DNA which hybridizes to the labeled probe.

25. A method of isolating DNA which is homologous to a gene contributing to senescence in an organism, comprising the steps of:

a) generating a genomic DNA library from the organism of interest;

b) contacting the library with a labeled probe comprising DNA encoding a gene isolated by the method of Claim 17, under conditions of low stringency; and

c) isolating from the library DNA which hybridizes to the labeled probe.

26. Isolated DNA consisting essentially of a gene contributing to senescence in an organism, isolated by the method of either of Claim 24 or Claim 25.

27. A gene contributing to senescence, isolated from the mutant yeast cell of Claim 7.

28. A protein encoded by the gene of Claim 27.

29. A protein encoded by DNA of Claim 26.

30. A mutant SIR4 gene, consisting essentially of a SIR4 gene having a stop at codon 1237.

31. A gene encoding a protein having the amino acid sequence encoded by a SIR4 gene having a stop at codon 1237.

32. DNA which hybridizes under high stringency to the gene of Claim 30.

33. The protein encoded by the gene of Claim 30 or 31.

34. The UTH1 gene having the nucleotide sequence of SEQ. ID. No. 1.

35. DNA which hybridizes under high stringency conditions to the UTH1 gene having the nucleotide seqeunce of SEQ. ID. NO. 1.

36. A gene encoding a UTH1 protein having the amino acid sequence of SEQ. ID. NO. 2.

37 The UTH1 protein having the amino acid sequence of SEQ. ID. NO. 2.

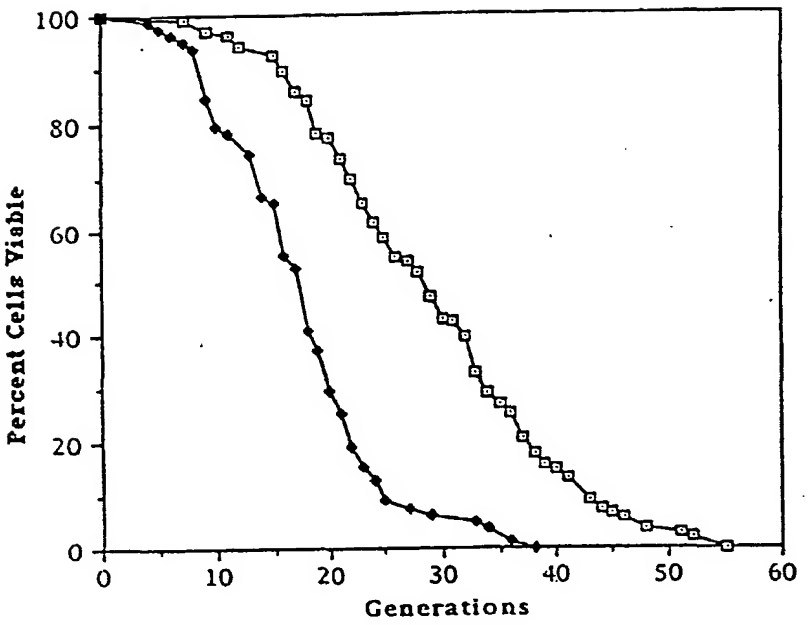
Percent Cells Viable
100
80
60
40
20
0
0
10
20
30
40
50
60
Generations

FIGURE 1

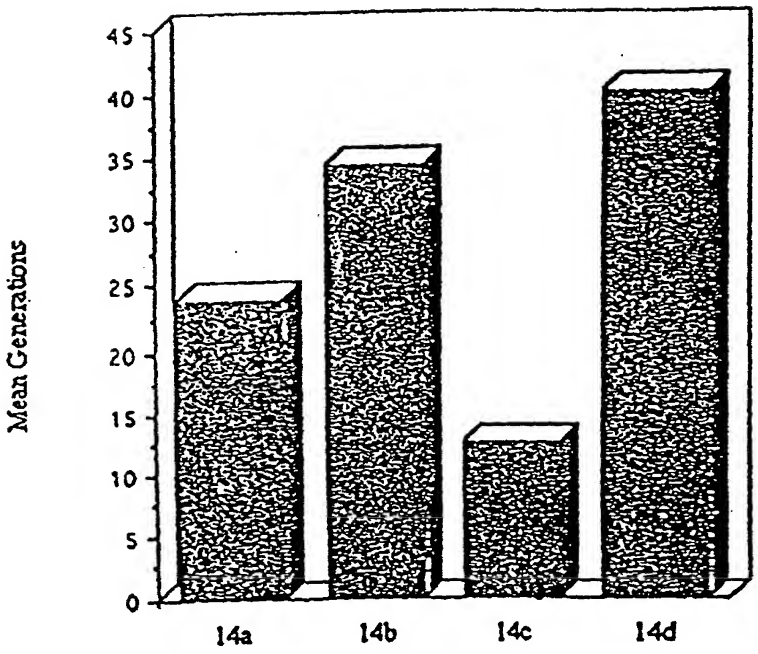

FIGURE 2

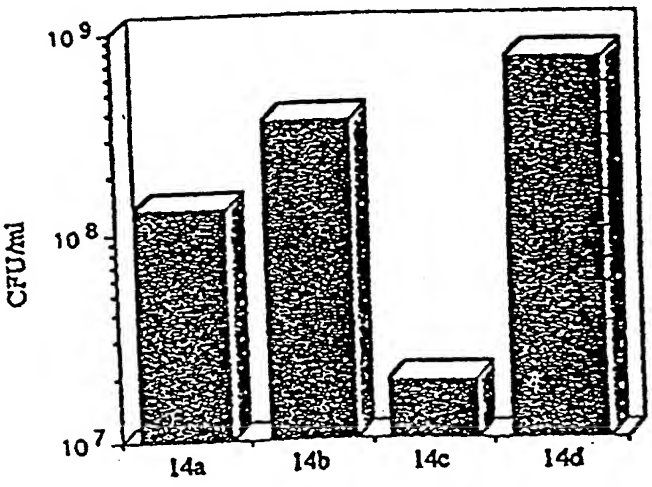

FIGURE 3

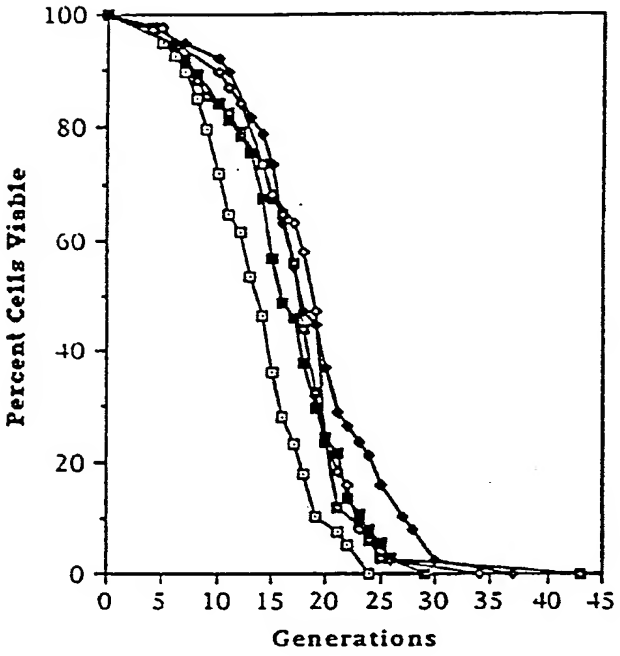
Percent Cells Viable
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45
Generations

FIGURE 4

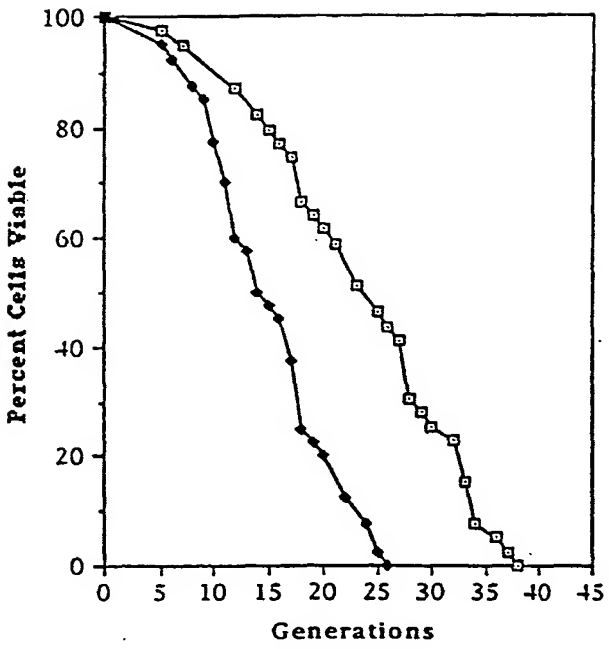
Percent Cells Viable
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45
Generations

FIGURE 5

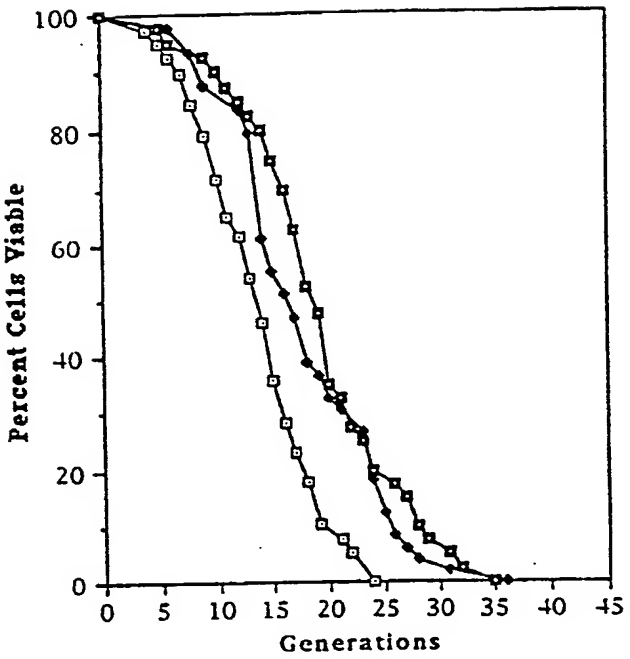
Percent Cells Viable
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45
Generations

FIGURE 6

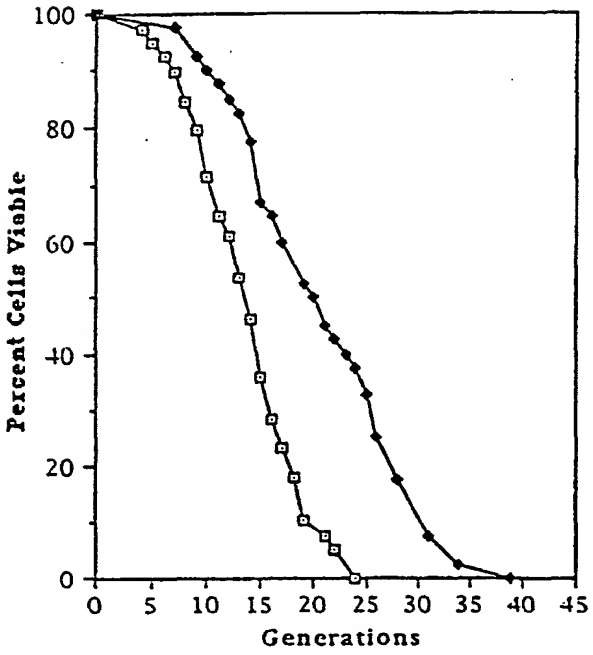
Percent Cells Viable
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45
Generations

FIGURE 7

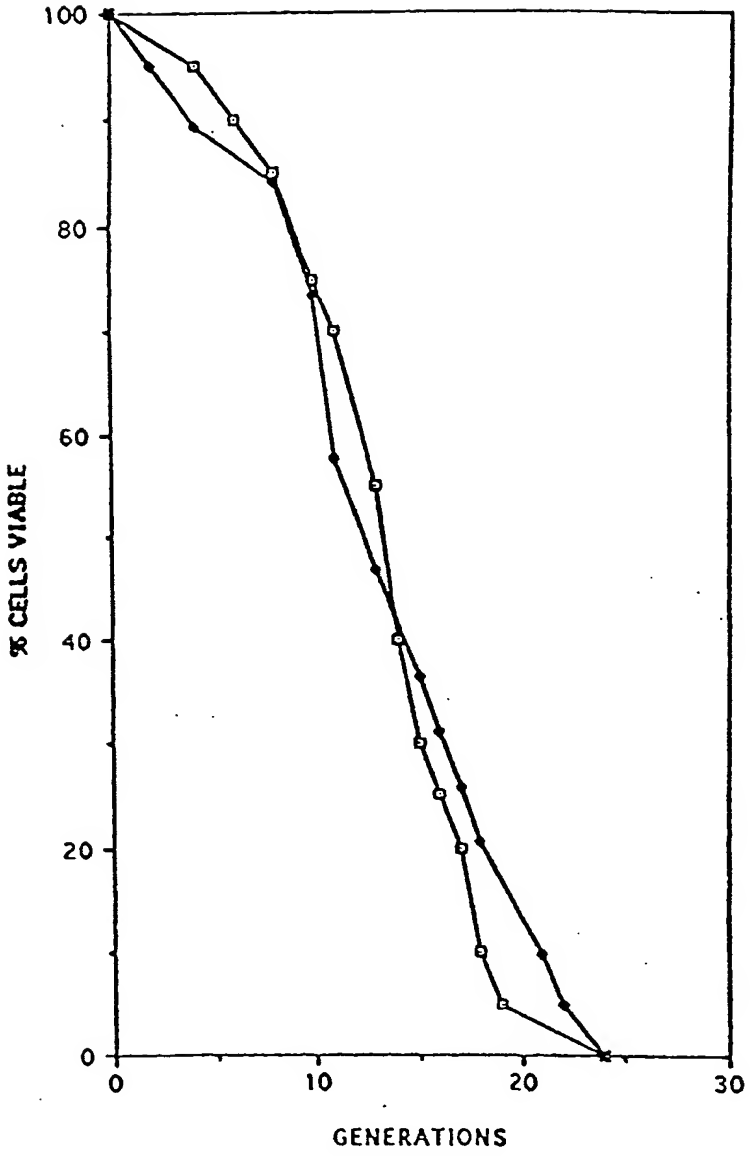

FIGURE 8

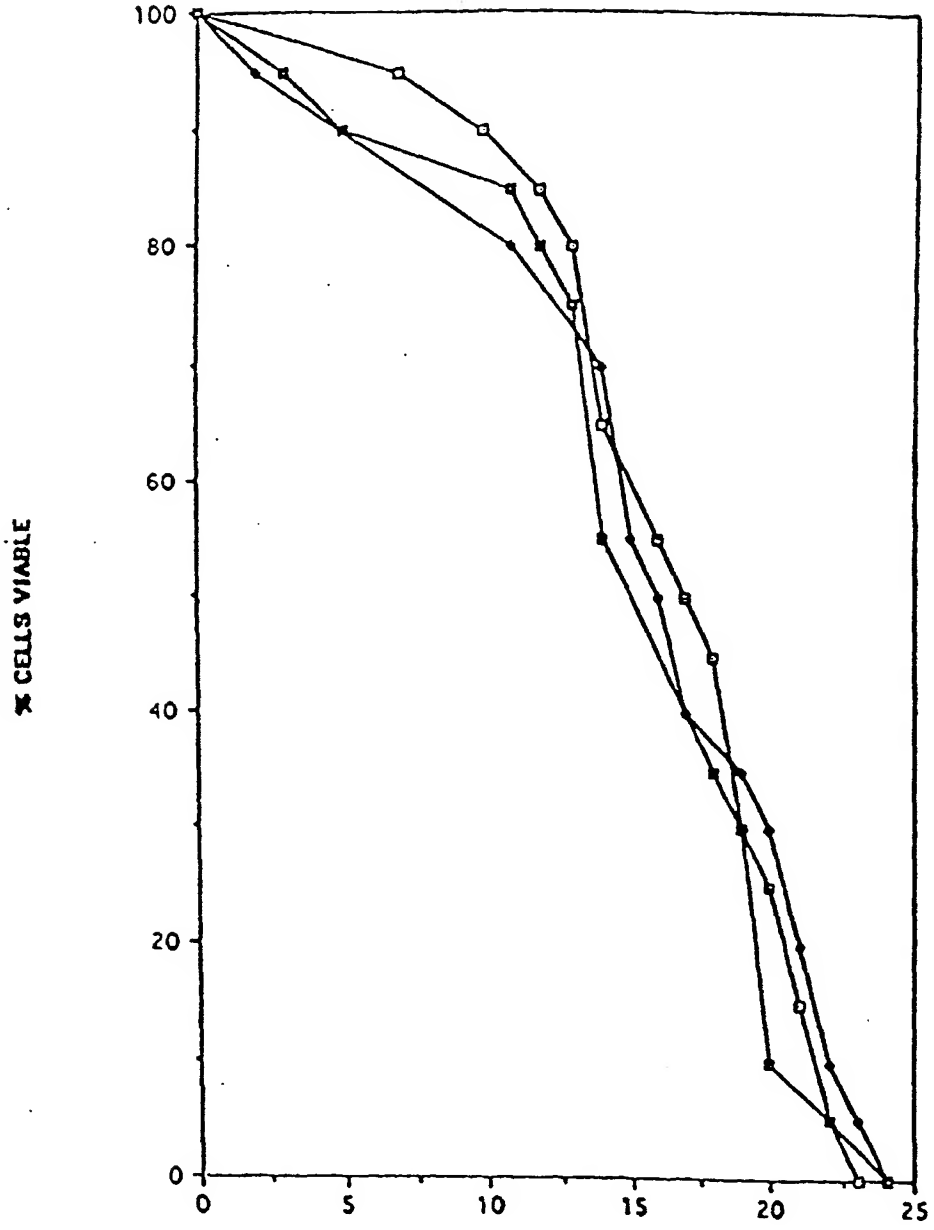

FIGURE 9

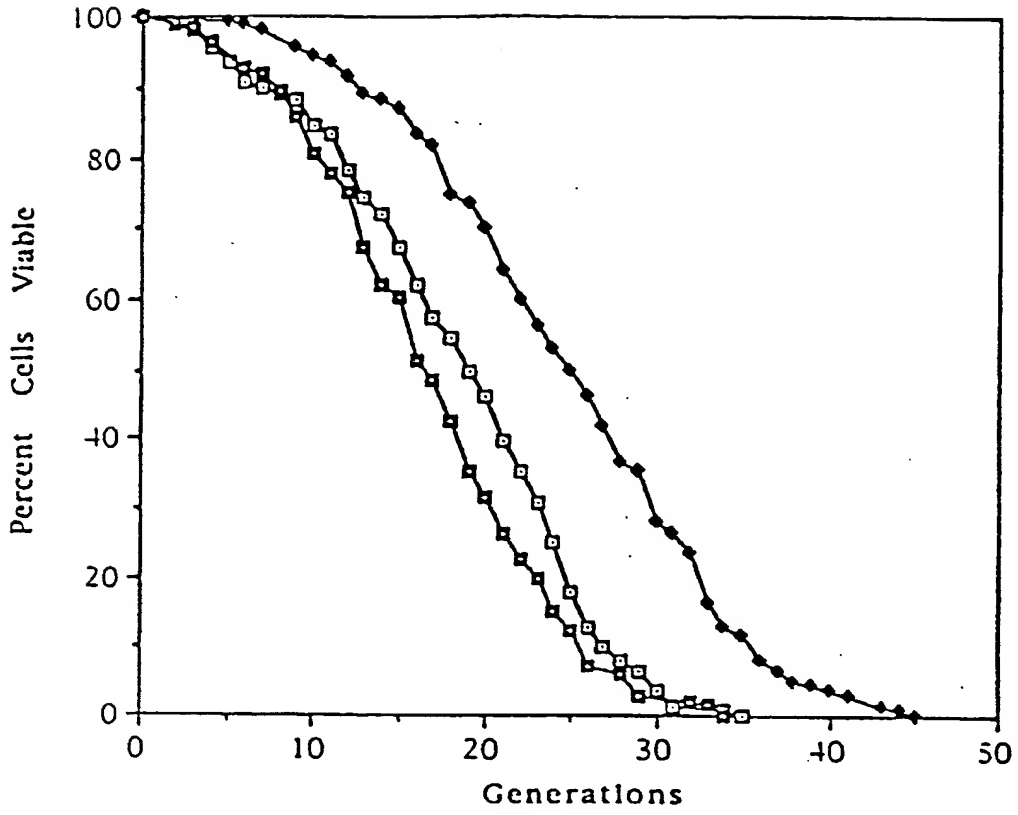
Percent Cells Viable
100
80
60
40
20
0
0
10
20
30
40
50
Generations

FIGURE 10

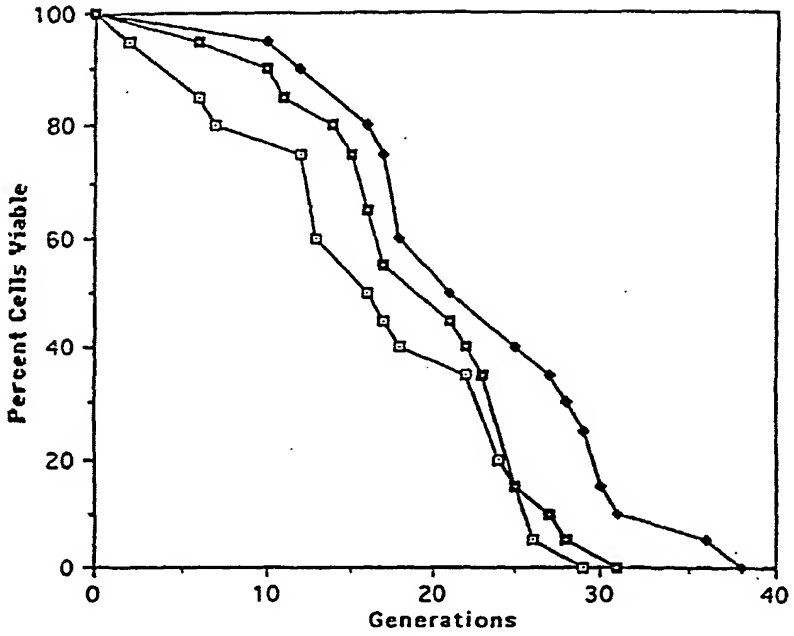
100
80
60
40
20
0
Percent Cells Viable
0
10
20
30
40
Generations

FIGURE 11

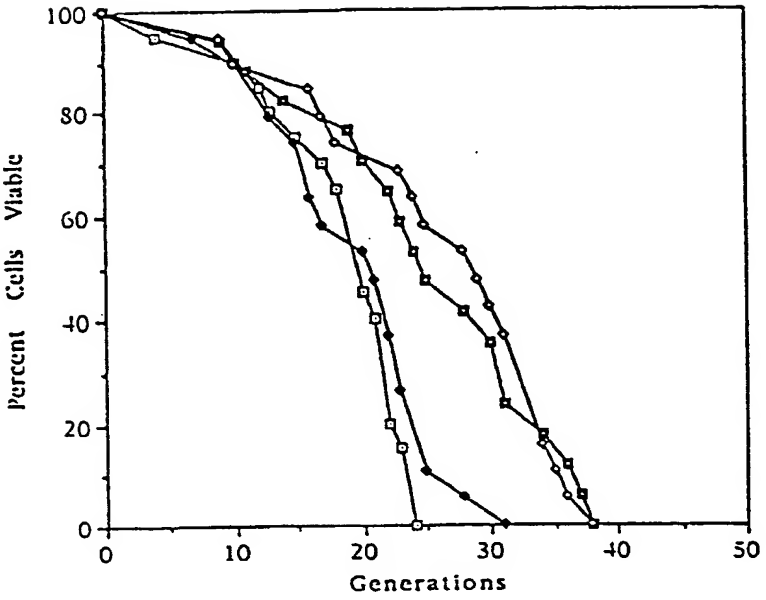

FIGURE 12

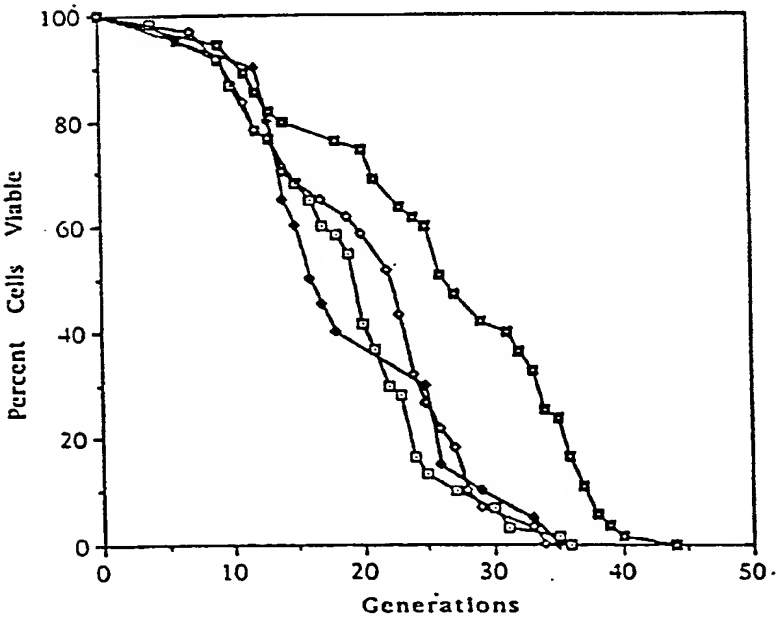

FIGURE 13

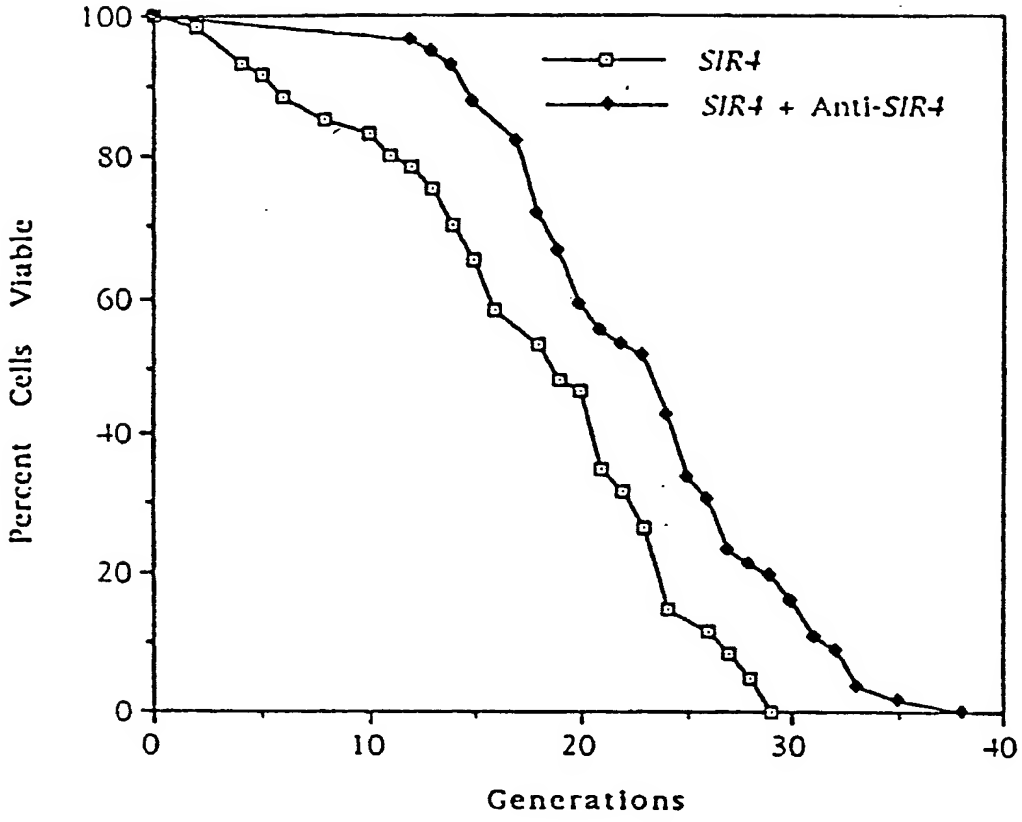
100
80
60
40
20
0
Percent Cells Viable
0
10
20
30
40
Generations
SIR4
SIR4 + Anti-SIR4

FIGURE 14

```
TGAAAAAGTG GAACTAGACC CCACGTCAGC GGGCCTAGGC CCTTCAATGT GTTAGAATAC      60

ACAGCGTGCC TAGTTCCTGG TGCCTGGATC TCGAGGCCGC GGCACTGGAA AAGCCCTTTC     120

TTTTCCAGAT CGGGAAACCT AATGAGTCCA TAAAAAGAAA TGTAGAGGTG GTGTTGACGT     180

TTTGCCGCTT TTGGGCAAGT AGGTCTTTCT GCACGGCCCG GCCCGGGTCG TGCGGAAAAA     240

GAAAAAAGCA GACAAAACAA AATTTTTCCT TTTTTTCGCC TTTGTTTCTC CTGATTCGGG     300

TATATAAGTG AATACCATCT A ATG TGT TTC CTT CTC GAG ACC TCG GCG TCT        351
                         Met Cys Phe Leu Leu Glu Thr Ser Ala Ser
                           1               5                  10

CCC AGA TCA AAG CTC AGC AAA GAT TTT AAA CCG CAA TTT ACG CTC CTT        399
Pro Arg Ser Lys Leu Ser Lys Asp Phe Lys Pro Gln Phe Thr Leu Leu
                    15                  20                  25

TCA TCG GTA ACT AAG AAG AAA AAA AAA AAA GTA CGA CCA CAC AAT TTC        447
Ser Ser Val Thr Lys Lys Lys Lys Lys Lys Val Arg Pro His Asn Phe
                30                  35                  40

CAG TGT ATT CAT TCC TTA AAC TTC GTT TAT TTT TTA TTC ATT CAT TCA        495
Gln Cys Ile His Ser Leu Asn Phe Val Tyr Phe Leu Phe Ile His Ser
            45                  50                  55

TTT TTA TTT GAA TAT AAC CAA CTA CTA GTC CTT CCT TTA AAC AAA AAT        543
Phe Leu Phe Glu Tyr Asn Gln Leu Leu Val Leu Pro Leu Asn Lys Asn
        60                  65                  70

TTA CCC TCC CTT AAT TTT TCA AGA AAT TCC AGT ATG AAA TTA TCC GCT        591
Leu Pro Ser Leu Asn Phe Ser Arg Asn Ser Ser Met Lys Leu Ser Ala
 75                  80                  85                  90

CTA TTA GCT TTA TCA GCC TCC ACC GCC GTC TTG GCC GCT CCA GCT GTC        639
Leu Leu Ala Leu Ser Ala Ser Thr Ala Val Leu Ala Ala Pro Ala Val
                95                 100                 105

CAC CAT AGT GAC AAC CAC CAC CAC AAC GAC AAG CGT GCC GTT GTC ACC        687
His His Ser Asp Asn His His His Asn Asp Lys Arg Ala Val Val Thr
            110                 115                 120

GTT ACT CAG TAC GTC AAC GCA GAC GGC GCT GTT GTT ATT CCA GCT GCC        735
Val Thr Gln Tyr Val Asn Ala Asp Gly Ala Val Val Ile Pro Ala Ala
        125                 130                 135

ACC ACC GCT ACC TCG GCG GCT GCT GAT GGA AAG GTC GAG TCT GTT GCT        783
Thr Thr Ala Thr Ser Ala Ala Ala Asp Gly Lys Val Glu Ser Val Ala
    140                 145                 150

GCT GCC ACC ACT ACT TTG TCC TCG ACT GCC GCC GCC GCT ACT ACC TCT        831
Ala Ala Thr Thr Thr Leu Ser Ser Thr Ala Ala Ala Ala Thr Thr Ser
155                 160                 165                 170

GCC GCC GCC TCT TCT TCC TCC TCT TCC TCT TCC TCC TCT TCC TCT TCT        879
Ala Ala Ala Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser
                175                 180                 185
```

FIGURE 15 (1 of 3)

```
TCC TCT GTT GGT TCT GGA GAT TTT GAA GAT GGT ACC ATT TCC TGT TCT        927
Ser Ser Val Gly Ser Gly Asp Phe Glu Asp Gly Thr Ile Ser Cys Ser
            190                 195                 200

GAT TTC CCA TCC GGA CAA GGT GCT GTC TCC TTG GAC TGG TTA GGT CTA        975
Asp Phe Pro Ser Gly Gln Gly Ala Val Ser Leu Asp Trp Leu Gly Leu
        205                 210                 215

GGC GGC TGG GCT TCC ATC ATG GAC ATG AAC GGT AAC ACC GCC ACC TCT       1023
Gly Gly Trp Ala Ser Ile Met Asp Met Asn Gly Asn Thr Ala Thr Ser
    220                 225                 230

TGT CAA GAC GGA TAC TAC TGT TCT TAC GCT TGT TCT CCA GGT TAC GCT       1071
Cys Gln Asp Gly Tyr Tyr Cys Ser Tyr Ala Cys Ser Pro Gly Tyr Ala
235                 240                 245                 250

AAG ACC CAA TGG CCT TCT GAA CAA CCT TCC GAT GGT AGA TCC GTT GGT       1119
Lys Thr Gln Trp Pro Ser Glu Gln Pro Ser Asp Gly Arg Ser Val Gly
                255                 260                 265

GGT TTA TAC TGT AAG AAC GGT AAA TTA TAC CGT TCC AAC ACC GAC ACT       1167
Gly Leu Tyr Cys Lys Asn Gly Lys Leu Tyr Arg Ser Asn Thr Asp Thr
            270                 275                 280

AAC AGT TTG TGT GTA GAA GGT CAA GGC TCT GCT CAA GCT GTT AAC AAG       1215
Asn Ser Leu Cys Val Glu Gly Gln Gly Ser Ala Gln Ala Val Asn Lys
        285                 290                 295

GTC TCC GGC TCC ATT GCT ATC TGT GGT ACC GAT TAT CCA GGT TCT GAA       1263
Val Ser Gly Ser Ile Ala Ile Cys Gly Thr Asp Tyr Pro Gly Ser Glu
    300                 305                 310

AAC ATG GTC GTT CCT ACC GTA GTT GGC GCT GGT TCC TCC CAA CCA ATC       1311
Asn Met Val Val Pro Thr Val Val Gly Ala Gly Ser Ser Gln Pro Ile
315                 320                 325                 330

AAC GTC ATC AAG GAG GAC TCC TAC TAT CAA TGG CAA GGT AAG AAG ACC       1359
Asn Val Ile Lys Glu Asp Ser Tyr Tyr Gln Trp Gln Gly Lys Lys Thr
                335                 340                 345

TCT GCC CAA TAC TAC GTT AAC AAC GCT GGT GTC TCT GTG GAA GAT GGT       1407
Ser Ala Gln Tyr Tyr Val Asn Asn Ala Gly Val Ser Val Glu Asp Gly
            350                 355                 360

TGT ATC TGG GGT ACT GAG GGT TCC GGT GTC GGT AAC TGG GCC CCA GTT       1455
Cys Ile Trp Gly Thr Glu Gly Ser Gly Val Gly Asn Trp Ala Pro Val
        365                 370                 375

GTC TTG GGT GCT GGT TAC ACT GAT GGT ATC ACT TAC TTG TCC ATC ATT       1503
Val Leu Gly Ala Gly Tyr Thr Asp Gly Ile Thr Tyr Leu Ser Ile Ile
    380                 385                 390

CCA AAC CCA AAC AAC AAA GAA GCA CCA AAC TTT AAC ATC AAG ATC GTT       1551
Pro Asn Pro Asn Asn Lys Glu Ala Pro Asn Phe Asn Ile Lys Ile Val
395                 400                 405                 410

GCC ACC GAT GGC TCT ACC GTC AAT GGT GCT TGC TCT TAC GAA AAT GGT       1599
Ala Thr Asp Gly Ser Thr Val Asn Gly Ala Cys Ser Tyr Glu Asn Gly
                415                 420                 425
```

FIGURE 15 (2 of 3)

```
GTC TAC TCT GGC TCT GGC TCT GAC GGT TGT ACT GTT TCA GTT ACT TCT      1647
Val Tyr Ser Gly Ser Gly Ser Asp Gly Cys Thr Val Ser Val Thr Ser
            430                 435                 440

GGT TCT GCT AAC TTT GTC TTC TAC TAGGCCTTTT TTCCTTGAAT ATTGCAAATA    1701
Gly Ser Ala Asn Phe Val Phe Tyr
        445                 450

AGCTTTTGCT AGTACTTTTT TTACTCCGTT CATTTTATGG TTTATTTTTC AATTAGTTCG    1761

TTTTTCCACA ATACAAAAAA ACACAGTCCT TTGTACTATC CCTTTTATTT CATTATTTTT    1821

TCTTTTTTAA GATACCACTA GATATTATCA TATATAGCAT ATTATATAAC ATAAAAAGTC    1881

AAGAAAAAAA ATGTTTTTAT CACTTTCTAT AACTGCATAT CTTTTTTTGC ATTTCGAATG    1941

ATTGC                                                               1946
```

FIGURE 15 (3 of 3)

ANNEX M3

International Application No: PCT/ US94/ 09351

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 12, line 12 of the description [1]

A. IDENTIFICATION OF DEPOSIT [1]

Further deposits are identified on an additional sheet ☐ [2]

Name of depositary institution [4]
American Type Culture Collection
12301 Parklawn Drive
Rockville, Maryland 20852 USA

Address of depositary institution (including postal code and country) [4]
12301 Parklawn Drive
Rockville, Maryland 20852 USA

Date of deposit [5]	Accession Number [6]
13 August 1993	74236

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European Patent is sought, the Applicant hereby informs the European Patent Office under European Rule 28(4) that, until the publication of the mention of the grant of the European Patent or until the date on which the European Application has been refused or is withdrawn or is deemed to be withdrawn, the availability of the biological material deposited with the American Type Culture Collection under Accession No. shall be effected only by the issue of a sample to an expert nominated by the requester in accordance with European Rule 28(5).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [3] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [8] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☑ This sheet was received with the international application when filed (to be checked by the receiving Office)

Virginia L Libby
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was _____
(Authorized Officer)

(January 1985)

Form PCT/RO/134 (January 1981)

PCT/US 94/09351

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/01 C12N15/11 C12N1/16 C07K14/395 C12Q1/02
C12Q1/68 //(C12N1/16,C12R1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABSTRACTS OF THE 92ND GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY 26-30 May 1992, page 230 N.P. D'MELLO ET AL.: 'Molecular analysis of a young-specific gene in the yeast Saccharomyces cerevisiae.' see abstract n. H-284 --- -/--	21,26,27

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search	Date of mailing of the international search report
3 January 1995	23 -01- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.264, no.24, 25 August 1989, BALTIMORE, MD US pages 14312 - 14317 NEJAT K. EGILMEZ ET AL. 'Specific alterations in transcript prevalence during the yeast life span' see abstract see page 14314, left column, paragraph 2 - right column, paragraph 1 see page 14315, left column, paragraph 1 see page 14315, right column, paragraph 2 - page 14316, left column, paragraph 1 see page 14316, right column, last paragraph - page 14317, left column, paragraph 1	9,13,15, 17,21,27
A	PROCEEDINGS OF A UCLA COLLOQUIUM. MOLECULAR BIOLOGY OF AGING, March 1989 pages 189 - 203 S. MICHAEL JAZWINSKI ET AL. 'Replication control and differential gene expression in aging yeast' see page 197, paragraph 2 - page 200, paragraph 1	9,13,15, 17
A	MECHANISMS OF AGEING AND DEVELOPMENT, vol.12, no.1, January 1980 pages 47 - 52 ILSE MÜLLER ET AL. 'Calendar life span versus budding life span of Saccharomyces cerevisiae' see page 47, paragraph 3 - page 48, paragraph 1 see page 48, paragraph 4 - page 50, paragraph 2	1,3-5,7
P,X	PROTEIN SEQUENCE DATABASE Accession number S38114; 3 May 1994 URRESTARAZU ET AL.	28,29,37

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